

## **Guidelines for the Research Use of Adjuvants**

The use of adjuvants in animal research studies of basic immunological phenomena, and in applied immunology, requires careful consideration. The apparent requirement for non-specific inflammation to elicit robust immunity obliges the investigator to evaluate the cost of potential, local and/or systemic pain and/or distress of the research animal due to the inflammation with the presumed scientific benefit to be gained from the experiment. The validity and applicability of the scientific knowledge gained must be tempered with acknowledgement that the use of potent inflammatory agents, particularly Complete Freund's Adjuvant (CFA), should be considered early during the development of the experimental design. Whenever possible alternatives to CFA should be used (1).

Adjuvants known to produce less intense inflammatory responses should be strongly considered as alternatives to CFA. These include TiterMax, Ribi Adjuvant System (RAS), Montanides, Syntex Adjuvant Formulation (SAF), aluminum compounds (e.g., alum), subcutaneously-implanted chambers (5) and others. In many situations these alternatives are capable of eliciting sufficient cellular and humoral antibody responses with fewer side effects than those commonly seen with CFA. Information on alternative adjuvants is available on-line (see references).

### **Complete Freund's Adjuvant**

CFA, a water-in-oil emulsion containing heat-killed mycobacteria or mycobacterial cell wall components, is an effective means of potentiating cellular and humoral antibody response to injected immunogens. Adjuvant activity is a result of sustained release of antigen from the oily deposit and stimulation of a local innate immune response resulting in enhanced adaptive immunity. An essential component of this response is an intense inflammatory reaction at the site of antigen deposition resulting from an influx of leukocytes and their interaction with antigen. The use of CFA is an important biologic resource for investigators, which should be used responsibly and with care to avoid or minimize the adverse effects of excessive inflammation. CFA may result in local inflammation and granulomatous reactions at the site of injection. CFA used improperly or excessively can cause significant side effects such as chronic inflammation, skin ulceration, local abscess or tissue sloughing. Other complications observed following CFA use are diffuse systemic granulomas secondary to migration of the oil emulsion, adjuvant-related arthritis, and chronic wasting disease.

The following guidelines are directed toward the elimination or minimization of complications secondary to immunization with CFA. Utilization of: a) sterile technique in the preparation of antigen-adjuvant emulsions; b) aseptic preparation of the injection site; c) appropriate injection technique; d) appropriate routes and sites of administration; e) adequate separation of injection sites; and f) use of smaller volumes at each injection site have all proven efficacious in the elimination of post-immunization complications.

Antigen preparations should be sterile and, ideally, isotonic, pH neutral, and free of urea, acetic acid, and other toxic solvents. Antigens separated using polyacrylamide gels should be further purified whenever possible or the amount of polyacrylamide gel should be reduced by careful trimming, to minimize the amount of secondary inflammation/irritation from gel fragments. Millipore filtration of the antigen prior to mixing it with the adjuvant is recommended to remove as much extraneous microbial contamination as possible.

The mycobacteria in CFA is resuspended by vortexing or shaking the ampule or vial. The CFA is then removed from the ampule or vial using sterile technique. Although approaches may vary, one part or less of CFA to one part antigen (v/v) has been recommended (1). Care should be taken to prevent introducing bubbles of air when mixing the CFA/antigen emulsion.

Although formulations of CFA containing 0.5 mg/ml mycobacterial concentration are commercially available and have been used successfully by many researchers, concentrations of < 0.1 mg/ml are recommended to minimize the inflammation and necrosis observed with higher concentrations (2). Use of greater concentrations than commercially available are not recommended unless scientifically justified and approved by the institutional ACUC. In addition, use of preparations containing disrupted mycobacterial cells rather than whole, intact bacilli may prove desirable because of the inability of the latter to be distinguished histologically from live, acid-fast cells.

Prior to immunization, the injection site should be clipped and surgically scrubbed to minimize the chance of bacterial contamination. Experience has demonstrated that the use of injection volumes and sites appropriate for the species, size of the animal, and experimental goal (Table 1) produce favorable results while minimizing undesirable side effects (3, 4). Some routes of injection may potentially be less disruptive to the animal than other routes (e.g., subcutaneous injection vs. foot-pad administration). Whenever possible the least invasive methodology required to accomplish the experimental goal should be utilized. Intra-dermal and footpad injections should be avoided unless scientifically justified. Separation of multiple injection sites by a distance sufficient to avoid coalescence of inflammatory lesions; and a period of 2 weeks between subsequent inoculations are recommended. In addition to the route of administration, the site of injection should be chosen with care to avoid areas that may compromise the normal movement or handling of the animal (e.g., intradermal injections in the scruff of the neck of a rabbit).

When raising hyperimmune serum, CFA is usually only necessary for the initial immunization, while incomplete Freund's adjuvant, which lacks mycobacterium, is the adjuvant of choice for subsequent immunizations. CFAs containing either *M. butyricum* or *M. tuberculosis* H37Ra (an avirulent strain) are commercially available. Additional information about CFA use is available on-line (see references).

### **Route of Administration**

#### **Footpad Immunization:**

Utilizing the footpad for immunization of small rodents may be necessary in particular studies where the isolation of a draining lymph node, as a primary action site, is required. The well-being of subject animals should be addressed by procedures such as limiting the quantity of adjuvant-antigen solution injected into the footpad, the use of only one foot per experimental animal, and housing on soft bedding rather than screens. In instances where there is no evidence indicating a specific requirement for footpad inoculation, this technique should not be used for routine immunization of rodents. If scientific justification is provided, the recommended maximum footpad injection volumes are 0.01-0.05 in mice and 0.10 ml for rats (1). Rabbits should not be immunized in their feet, because they do not have a true footpad.

#### **Peritoneal Exudate:**

The production of rodent peritoneal exudate by the intraperitoneal administration of antigen and adjuvant is a widely recognized valid scientific procedure for obtaining high titer reagent.

Undesirable side effects of painful abdominal distention and the resulting distress can be avoided by daily monitoring and relief of ascites pressure, or termination of the experiment. Intraperitoneal injections of CFA-antigen emulsions should normally be limited to less than 0.2 ml in mice (6).

### Post-injection Observations and Treatments

Post-inoculation monitoring of animals for pain and distress or complications at the injection sites is essential and should be done daily for a minimum of four weeks or until all lesions have healed. Supportive therapy may include topical cleansing, antibiotics, and use of an analgesic. Although analgesics are not routinely required, the use of narcotic agonists, mixed agonist-antagonists, or other species-appropriate agents should be considered, taking into account the research objective, if overt pain or distress is observed. Steroidal or non-steroidal anti-inflammatory agents must be used with caution due to their direct impacts on immunological processes.

### Personnel Safety

Handling of adjuvants that contain mycobacterial products can be an occupational hazard to laboratory personnel. Reports of accidental needle punctures in humans have been associated with clinical pain, inflammatory lesions, and abscess formation in tuberculin-positive individuals. Tuberculin-negative individuals have tested positive in subsequent tuberculin tests after accidental CFA exposure (7). Safety glasses should be worn to avoid accidental splashing of CFA in the eyes.

### Other Considerations

Scientists preparing antigens for in vivo administration in conjunction with adjuvants should be aware of the potential presence of contaminating substances and other characteristics of the injectate which may have additive inflammatory effects. Judicious use of adjuvant may be abrogated by failure to consider sterility of preparations, excessive vehicle pH, or the presence of by-products of purification such as polyacrylamide gel fragments. Care should be taken to consider and eliminate additional inflammatory stimuli whenever possible.

**Table 1. Recommended Volume of CFA-Antigen Emulsion (CFA-AE) per Site and Route of Administration**

Species	Subcutaneous	Intradermal	Intraperitoneal	Footpad	Intramuscular
Mouse	≤0.1 ml	*	≤0.2 ml	≤0.05 ml**	≤0.05 ml
Rat	≤0.1 ml	≤0.05 ml**	≤0.5 ml	≤0.1 ml**	≤0.1 ml
Rabbit	≤0.25 ml	≤0.05 ml**	*	*	≤0.25 ml***
Goat/Sheep	≤1.0 ml	≤0.1 ml**	*	NA	≤0.5 ml

\* Not recommended

\*\* Only When Justified

\*\*\* Only One Limb Recommended Without Justification

NA: Not applicable

**References:**

1. Jackson, L.R., and J.G. Fox. 1995. Institutional Policies and Guidelines on Adjuvants and Antibody Production. *ILAR Journal* 37(3):141-150.
2. Broderon, J. R. 1989. A Retrospective Review of Lesions Associated with the use of Freund's Adjuvant. *Lab. Anim. Sci.* 39:400-405.
3. Grumpstrup-Scott, J., and D. D. Greenhouse. 1988. NIH Intramural Recommendations for the Research use of Complete Freund's adjuvant. *ILAR News* 30(2):9.
4. Stills, H. F., and M. Q. Bailey. 1991. The use of Freund's Complete Adjuvant. *Lab Animal* 20(4):25-31.
5. Clemons, D. J., C. Besch-Williford, E. K. Steffen, L. K. Riley, and D. H. Moore. 1992. Evaluation of Subcutaneously Implanted Chamber for Antibody Production in Rabbits. *Lab. Anim. Sci.* 42(3):307-311.
6. Toth, L. A., A. W. Dunlap, G. A. Olson, and J. R. Hessler. 1989. An Evaluation of Distress Following Intraperitoneal Immunization with Freund's Adjuvant in Mice. *Lab. Anim. Sci.* 39(2):122-126.
7. Chapel, H. M., and August, P. J. 1976. Report of Nine Cases of Accidental Injury to Man with Freund's Complete Adjuvant. *Clin. Exp. Immunol.* 24:538-541.

**Websites:**

**Adjuvants and Antibody Production:**

<http://www.nal.usda.gov/awic/pubs/antibody/>

<http://research.uiowa.edu/animal/?get=adjuvant>

[http://www.ccac.ca/en/CCAC\\_Programs/Guidelines\\_Policies/GDLINES/Antibody/antibody.pdf](http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/Antibody/antibody.pdf)

**CFA:**

<http://www.research.sunysb.edu/research/animforms/ivpolycl.doc>

[http://medschool.mc.vanderbilt.edu/oor/iacuc/php\\_files/freund.php](http://medschool.mc.vanderbilt.edu/oor/iacuc/php_files/freund.php)

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